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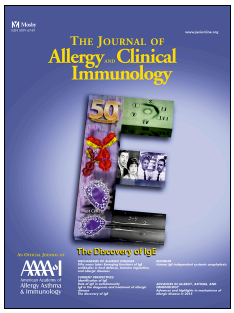

Differential expression of functional chemokine receptors on human blood and lung group 2 innate lymphoid cells (ILC2s)

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Differential expression of functional chemokine receptors on human blood and lung group 2 innate lymphoid cells (ILC2s).

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Capsule Summary: Differential expression of chemokine receptors on lymphocytes enables tissue and disease specific recruitment and activation of various lymphocytes. We have shown that human ILC2s display a different subset of functional chemokine receptors when isolated from blood or lung tissue.

Key Words: ILC2; chemokine receptor; T-cells.

Abbreviations

ILC2: group 2 innate lymphoid cell

LTi: lymphoid tissue inducer cell

NK cell: natural killer cell

Th-cell: T-helper cell

PBMCs: Peripheral blood mononuclear cells

MFI: mean fluorescence intensity

CCR: chemokine receptor (CC motif)

CXCR: chemokine receptor (CXC motif)

Main text: words

To The Editor,

Innate lymphoid cells (ILCs), primarily found at mucosal barriers, provide immediate protection against the establishment and spread of infection. ILCs have been divided into three subsets analogous to helper T (Th) cells (1); ILC1, ILC2 and ILC3, ILC2s are similar to Th2 cells and express IL-4, IL-5 and IL-13 and were initially identified as a non-T, non-B cell source of type 2 cytokines (2). They are found in the blood, gut, skin and lung where they contribute to host defence. Upon activation, ILCs rapidly produce a large quantity of cytokines and other mediators, which attract and activate other inflammatory cells. In various models of airway disease ILC2 numbers have been shown to increase with allergen challenge leading to a significant increase in type 2 inflammatory cytokines (3). Recent studies have demonstrated the existence of a complex interplay between lung epithelial cells and ILC2s that is required for asthma persistence in a mouse model. Furthermore, human studies have suggested that ILC2s provide the key link between viral infection and airway inflammation leading to asthma exacerbations (4).

ILC2s are produced from precursor cells in the bone marrow and ILC precursors have been identified in human blood, however there is debate about how ILCs populate the adult tissue with some studies indicating that they are predominantly tissue resident cells (5). An important question in ILC biology is to understand the mechanisms by which both progenitor and mature cells are recruited to the peripheral tissues such as the lung.

A significant class of cell surface receptors known to be important for immune cell migration are the chemokine receptors (6). Importantly, upregulation of key chemokines has been observed in the bronchial biopsies of asthmatic patients following allergen challenge. Due to this there has been much interest in the possibility of developing antagonists, which inhibit receptor activation, to prevent unwanted cells from being recruited to sites of inflammation such as the lung during asthma exacerbation (7). Using flow cytometry we assessed the expression of chemokine receptors on T-cells and ILC2s in both human blood and lung samples. For full methods please see online supplement. The mean data are represented in Figure 1. (11 blood donors and 5 lung samples). The pattern of receptor expression broadly agreed with previous reports for CD3+ T-cells. A smaller subset of receptors tested were readily detectable on ILC2s. These data were reflected at the RNA level as confirmed using real-time PCR probes to detect the different chemokine receptors in cDNA synthesised from RNA isolated from fresh ILC2 and T-cells (Supplemental Figure 4).

Although highly detectable on blood derived T-cells, CCR7 was not detected on ILC2s isolated from the blood or lung. CCR7 expression has previously been described on

lymphocyte precursors within the bone marrow but is only maintained on mature ILC1 and ILC3s, in particular subsets of ILCs found in the spleen and lymph nodes (8). Our data suggests that CCR7 does not play a role in the trafficking of mature ILC2s in the blood or lung tissues. CXCR5 and CXCR6 were only expressed on a small subset of blood T-cells and even fewer (<5%) lung T-cells. Furthermore, <5% of ILC2s displayed CXCR5 or CXCR6 receptor in either compartment. These results are consistent with the notion that CXCR5 is largely involved in B-cell homing and that CXCR6 is important for the retention of T-cells in the liver, and for the emigration of ILC3s from the bone marrow to the small intestine (8). CCR9, thought to direct cells to the small intestine was only detectable on around 10% of ILC2s in lung tissue.

Several receptors were significantly (p <0.05) upregulated in lung tissue derived ILC2s compared to the cells isolated from the blood including CCR3 and CXCR4 (Figure 4). CCR3 in combination with CCR4 has been shown in multiple T-cell studies (7) to regulate recruitment to the lung. Furthermore, the potent inflammatory ligand (CXCL12) acting via CXCR4 appears to coordinate with CCL11 activation of CCR3 and CCL22 stimulation of CCR4 to recruit lymphocytes to the lung and generate an inflammatory reaction (7). Our data indicate that a similar mechanism could be used to activate or recruit ILC2s to the airways thereby driving inflammation.

The receptors displayed on the highest proportion of ILC2s isolated from both blood and lung tissues were CCR2, CCR4, CCR5 and CCR6. We therefore wished to determine if these receptors could be used to activate blood ILC2s via various chemokine ligands. Since ILC2s are only found as a low percentage of human PBMCs traditional chemotaxis assays would have been difficult to reliably perform. We therefore used an actin polymerization assay as a marker of receptor activation. The ligands chosen for this assay (Supplemental table 2) have all been detected in the lung (7) and were based on their receptor specificity. The stimulation time was optimized to give the largest signal window (Supplemental Figure 5). Both ILC2s and T-cells displayed ligand-dependent increases in their F-actin content following 10sec stimulation with the chemokines (Figure 2). Due to the short duration of the assay it is unlikely that these effects are through indirect activation of other immune cells. The potency for each ligand was similar between the T-cells and ILC2s however, the maximal responses were significantly (p <0.05) different for all but CCL2 stimulation of CCR2, perhaps indicating differences in the number of the receptors expressed on each cell type. Comparing the response of ILC2s to each ligand (Figure 2E and Supplemental table 2) reveals that the strongest response is seen via CCR4, with CCR5 and CCR6 showing similar lower levels of activation and CCR2 an intermediary response. These values may reflect differences in receptor number on the ILC2 cell surface and may correspond to their roles in cell activity

and tissue recruitment as it is thought that a higher receptor number is required to achieve cell chemotaxis than. In T-cells CCR5 does not direct tissue specific recruitment but is required in combination with different panels of receptors to enable migration (7). Therefore it may be that initial activation of ILC2s by the more highly expressed receptors occurs before chemotaxis is enhanced through the binding of CCR5 specific ligands

Given that a higher proportions of "activated" IL-5^{+}, IL-13^{+} ILC2s have been shown to correlate with asthma severity (9) and that mouse models of asthma demonstrate that an increase in ILC2 number is sufficient for airway hyper responsiveness, targeting the activation and recruitment of these cells is an attractive treatment strategy. Our data provide new insight into the potential mechanisms by which ILC2s may be recruited or activated in the human blood and lung, and may therefore allow rational selection of future therapeutics targeting ILC2s in airway disease.

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177 **Figure Legends**

178

179 **Figure 1: ILC2s and T-cells isolated from human lung display different**
180 **chemokine receptors to those derived from blood. A**) Percentage of $CD3^+$ T-cells
181 from blood (white) and lung tissue (grey) expressing the indicated chemokine
182 receptors. **B)** Percentage of ILC2s from blood (white) and lung (red) expressing the
183 indicated chemokine receptors. Data is mean ± standard error of the mean (SEM) of
184 11 (PBMC) and 5 (lung) independent donors.

185

186 **Figure 2: ILC2s respond to various chemokine receptor ligands in a dose**
187 **dependent manner.** Activation of A) CCR2, B), CCR4, C) CCR5, D) CCR6
188 receptors on ILC2s and T-cells was determined following 10s stimulation with a
189 range of concentrations of the indicated ligands. E) Normalised ILC2 response for
190 each receptor/ligand combination. All data is mean ± SEM from 5 independent
191 experiments.

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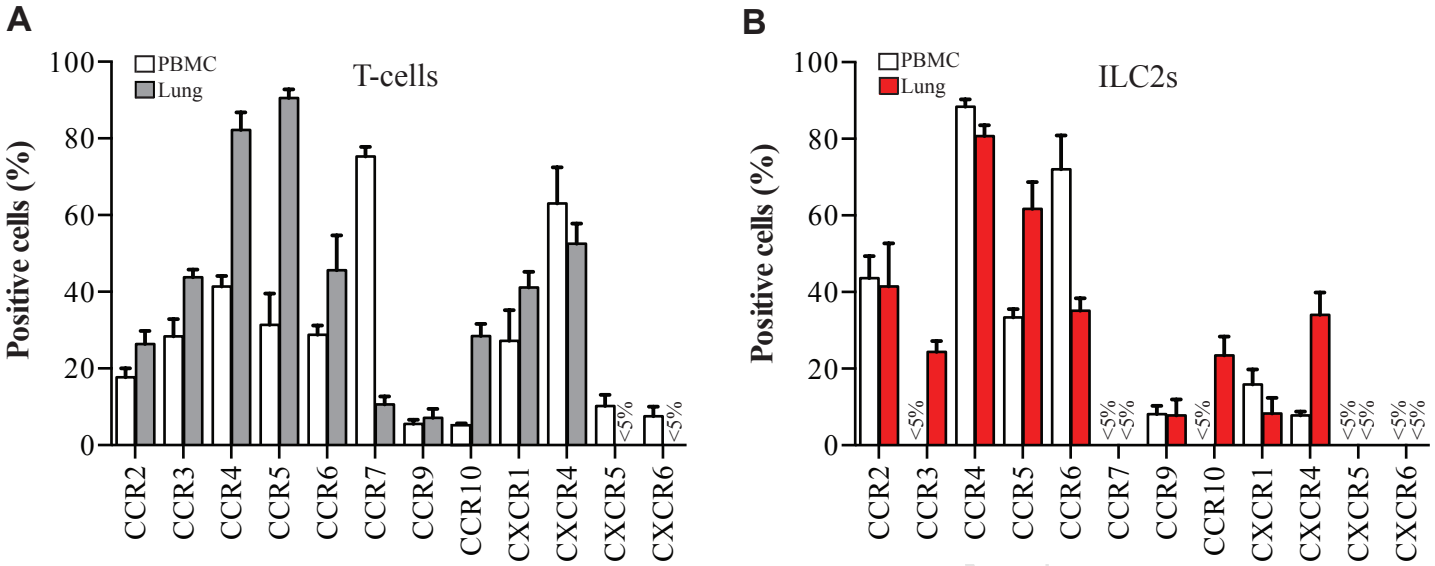

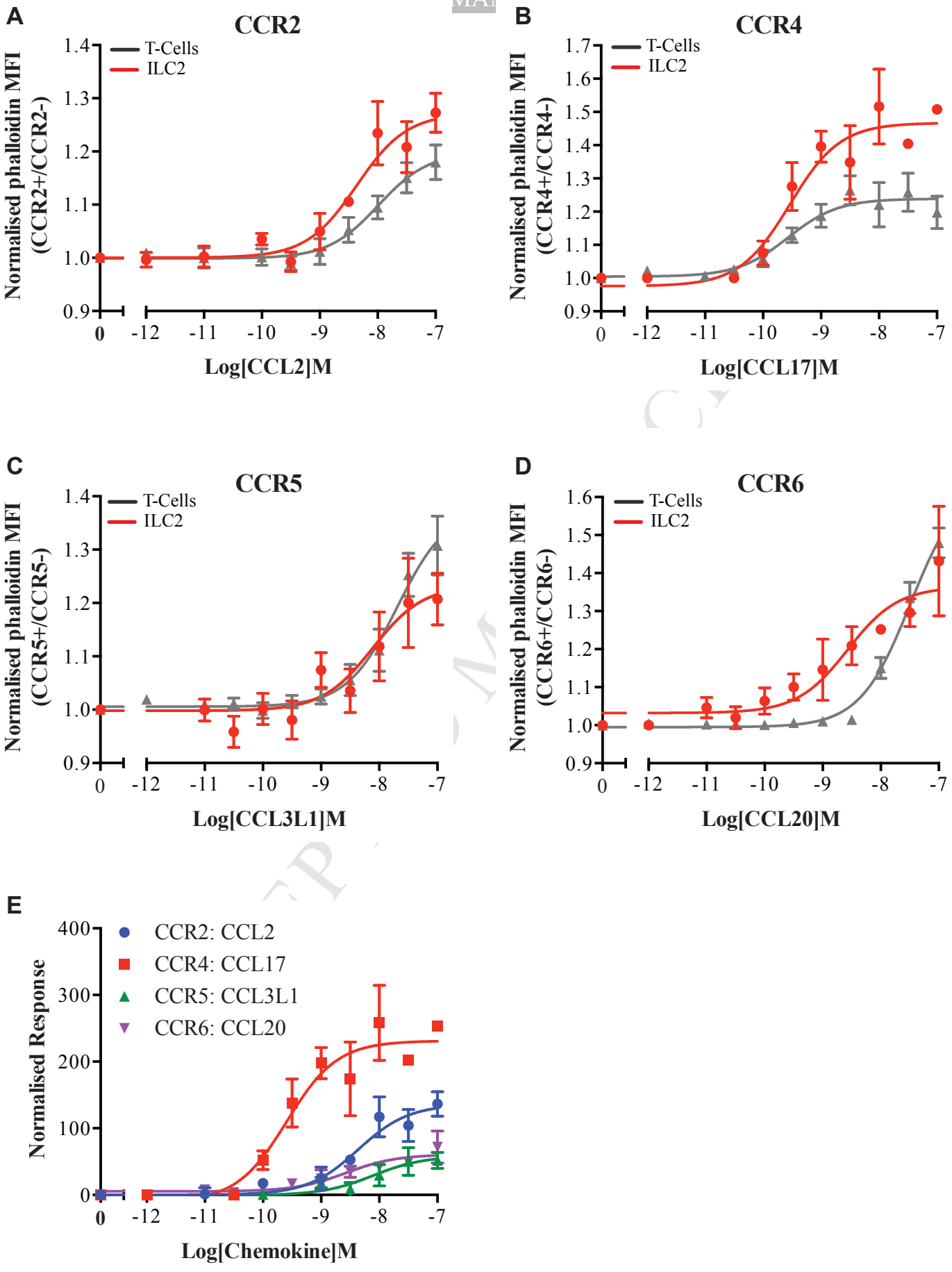
A
CCR2
T-Cells
ILC2
Normalised phalloidin MFI
(CCR2+/CCR2-)
Log[CCL2]M
B
CCR4
T-Cells
ILC2
Normalised phalloidin MFI
(CCR4+/CCR4-)
Log[CCL17]M
C
CCR5
T-Cells
ILC2
Normalised phalloidin MFI
(CCR5+/CCR5-)
Log[CCL3L1]M
D
CCR6
T-Cells
ILC2
Normalised phalloidin MFI
(CCR6+/CCR6-)
Log[CCL20]M
E
CCR2: CCL2
CCR4: CCL17
CCR5: CCL3L1
CCR6: CCL20
Normalised Response
Log[Chemokine]M

Supplemental Methods

PBMC isolation. Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood samples taken from healthy, non atopic subjects with no evidence of parasitosis recruited at University Hospitals of Leicester NHS Trust, Leicester, UK who had provided informed written consent, under ethics (REC reference number 08/H0406/189) approved by the Leicestershire, Northamptonshire and Rutland ethics committee. The heparinised blood was mixed with an equal volume of PBS and centrifuged (800*g*, 20min, 22^{o}C) over Lymphoprep (Stem Cell Technologies, Cambridge UK). Isolated PBMCs were washed in PBS containing 2% FBS (Thermofisher, Paisley, UK) before counting using a haemocytometer and trypan blue stain to enable resuspension at the concentration required for further experiments as described below.

Isolation of cells from lung tissue. Lung cells were isolated from healthy lung resection material taken from patients who had provided informed written consent and were undergoing lung surgery. This material was ethically obtained at University Hospitals of Leicester NHS Trust, under the auspices of the Midlands lung tissue consortium (REC reference number 07/MRE08/42). Lung tissue bathed in DMEM containing 2% FBS was enzymatically digested for 75 mins at 37^{o}C using hyaluronidase (0.75mg/ml, H3506, Sigma, UK) and collagenase (0.75mg/ml, C2674, Sigma) within 1h of resection. The digested tissue was filtered by sequentially passing through a 100µm then a 50µm gauze, and washed twice with DMEM containing 2% FCS (centrifuging at 230*g* for 8 mins, 4°C). Cell number was determined using haemocytometer and Kimura stain before washing in the absence of serum and resuspending in PBS at a concentration of 1x10^{7}/100µl for flow cytometric analysis as described below.

Analysis of cell surface markers by flow cytometry. For each condition tested 100µl (1x10^{7} cells) of lung tissue cells were first stained for 20mins with Zombie Aqua fixable viability kit following the manufacturer's protocol (Biolegend, London, UK). Following centrifugation (200*g* 10min, 4^{o}C) the stained cells were then resuspended in 100µl brilliant violet staining buffer (Beckton Dickinson, Oxford, UK). PBMCs were adjusted to a concentration 1x10^{6}/100µl prior to cell surface staining. Cells were first incubated with Human Trustain (Biolegend, London, UK) at room temperature for 15min to block Fc receptors. Monoclonal antibodies: FITC conjugated lineage cocktail (α-CD2, α-CD3, α-CD14, α-CD16, α-CD19, α-CD56, α-CD235a), EF450 conjugated α-CD3 (UCHT1) (both Affymetrix, Cheshire, UK) BV605 conjugated CD123 (6H6), AF647 conjugated α-CD294 (BM16), α-CCR2 (CD192; K036C2), α-CCR4 (CD194; L291H4), α-CCR5 (CD195; JF418F1), α-CCR6 (CD196;

G034E3), α-CCR7 (CD197; G043H7), α-CCR9 (CD199; L053E8), α-CXCR1 (CD181; 8F1/CXCR1), α-CXCR3 (CD183; G025H7), α-CXCR4 (CD184; 12G5), α-CXCR5 (CD185; J252D4), α-CXCR6 (CD186; K041E5) all PE/Cy7 conjugated, PE conjugated α-CCR3 (CD193; 5E8) and α-CCR10 (6588-5) (all Biolegend, London, UK) were then added for a further 20min incubation at room temperature. For lung tissue derived cells BV785 conjugated α-CD45 (HI30, Biolegend) was also added. Cells were then fixed and any red blood cells lysed through the addition of a one-step fix and lyse solution for 20min at room temperature (Affymetrix) before centrifugation at 300*g* 10min 4°C and resuspension in PBS supplemented with 2% FBS for acquisition on a four laser Attune NxT flow cytometer using NxT software v2.2 (Life Technologies). Compensation settings were determined using unstained cells and UltraComp eBeads™ compensation beads (Affymetrix). Following the exclusion of doublets (Supplemental Figure 1A), lymphocytes were selected based on their light scatter properties, (Supplemental Figure 1B). From the selected lymphocyte population both $CD3^+$ T-cells (Supplemental Figure 1C) and $lineage^-$, $CD123^-$, $CD294^+$ ILC2s were identified (Supplemental Figure 1D & E). CD45 was added as an additional positive marker for identifying T-cell and ILC2 populations in lung tissue (Supplemental Figure 1G). Due to the observed bimodal distribution of expression, the $CD3^+$ and chemokine receptor positive population was used to determine the proportion of ILC2s also displaying each chemokine receptor (Supplemental Figure 2 and 3, histograms).

Real time PCR. T-cells and ILC2s were sorted from PBMCs prepared as for flow cytometric analysis using a FACS ARIAIII (BD) directly into TRIZOL LS (ThermoFisher.) RNA was isolated from the purified cell populations using the miRNeasy mini kit (Qiagen), followed by DNAse digestion (Thermofisher) and RNeasy minElute cleanup (Qiagen) according to the respective manufacturer's instructions. RNA integrity was assessed using a Bioanalyser (Agilent, Edinburgh UK), according to the manufacturer's instructions. Due to low cell number cDNA samples were synthesized and amplified using the Ovation V2 kit (NuGEN, Leek, Netherlands) as per the manufacturer's instructions. Real-time RT-PCR was performed using TaqMan Master mix II (Thermofisher) and TaqMan probesets (all FAM-MGB) listed in Supplemental table 1. The chemokine receptor signals were compared to an 18S VIC®-MGB rRNA endogenous control (Thermofisher) Cycling conditions were as follows: 50oC for 2 mins, 95oC for 10 mins, 95oC for 15s then 60oC for 1 min for 50 cycles on a QuantStudio 5 system (Thermofisher).

Measurement of cellular F-actin content following chemokine stimulation. Isolated PBMCs were adjusted to $1x10^7$cells/100 μl. The ability of various recombinant chemokines

(CCL2, CCL17, CCL3L1, CCL20, all Biotechne) to activate their cognate receptor was then tested through measurement of chemokine-induced increases in the filamentous (F)-actin content of both $CD3^+$ T-cells and ILC2s. The method has previously been demonstrated as a marker for T-cell activation (1-2) Briefly, 100µl of cells per 10-point dose response curve were taken, Fc receptors blocked and stained with FITC conjugated CD123 and lineage cocktail, AF647 conjugated CD294, EF450 conjugated CD3 and the required PE/cy7 conjugated chemokine receptor antibodies for 20min at room temperature. Cells were washed in PBS containing 2% FBS and then resuspended in pre-warmed (37^{o}C) RPMI 1640 (ThermoFisher) containing 2% FBS before stimulation for 10sec with the appropriate chemokine. The reaction was terminated by the addition of an equal volume of fixation buffer from the intracellular fixation and permeabilisation buffer set (Affymetrix) for 30min at room temperature. Cells were washed (1200*g*, 5min) 3 times with 1x permeabilisation buffer (from the intracellular fixation and permeabilisation buffer set Affymetrix) and stained with AF555 conjugated phalloidin (Life Technologies) (30min at room temperature) before washing and resuspension in PBS containing 2%FBS. Data was acquired using an Attune NxT cytometer running NxT software v2.2. Chemokine-induced changes in F-actin content were quantified as an increase in the mean fluorescence intensity (MFI) in the AF555 channel. To account for photobleaching and other non-specific effects on AF555 fluorescence the MFI for chemokine receptor positive cells was expressed as a fraction of that observed in the population not displaying the receptor for each sample.

Data analysis. Analysis of flow cytometry data was performed using FlowJo v10 (Tree star, Oregon, USA). Concentration-response curves were fitted using the three-parameter logistic equation in Graph Pad PRISM v6 (California, USA) to obtain EC_{50} values. Statistical analysis was performed using a two-way ANOVA and Tukey's multiple comparison test with a probability (p) <0.05 being considered significant.

Supplemental References

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Supplemental Figure Legends

Supplemental Figure 1: Gating strategy to identify ILC2s and T-cells from human peripheral blood (A-E) and Lung (F-K). A) Total PBMCs displayed with singlet gate overlaid. **B**) Lymphocytes (gated cells) selected from single cell population based on light scattering properties. **C**) T-cells identified as the $CD3^+$ population of single cell lymphocytes. **D**) $Lineage^-$, $CD294^+$ cells (boxed region) containing **E**) the $CD123^-$ ILC2s were also selected from the single cell lymphocyte population. **F**) Total cells displayed with singlet gate overlaid. **G**) Lymphocytes (gated cells) selected from single cell population based on light scattering properties. **H**) Live ($zombie\ aqua^-$), $CD45^+$ cells shown in gated region were selected and **I**) T-cells were identified as the $CD3^+$ population **J**) $Lineage^-$, $CD294^+$ cells (boxed region) containing **K**) the $CD123^-$ ILC2s were also selected from the live, $CD45^+$, single cell lymphocyte population.

Supplemental Figure 2: Chemokine receptor expression on T-cells and ILC2s in human blood. Representative flow cytometry data showing the expression of the indicated chemokine receptors on $CD3^+$ T-cells (left) and ILC2s (right). $Receptor^+$ gate determined using bimodal distribution observed on T-cell populations, numbers within gates indicate percentage cells.

Supplemental Figure 3: Expression of chemokine receptors on ILC2s and T-cells isolated from lung tissue. A) Representative flow cytometry data showing the expression of the indicated chemokine receptors on: $CD3^+$ T-cells (left) and ILC2s (right). $Receptor^+$ gate determined using bimodal distribution observed on T-cell populations numbers within gates indicate percentage cells.

Supplemental Figure 4: Quantification of chemokine receptor RNA expression in T-cells and ILC2s. Relative quantities of the indicated genes were calculated relative to 18S mRNA. Plots show mean values (±SEM) for T-cells (black boxes) and ILC2s (grey boxes) sorted from PBMCS obtained from 5 independent donors.

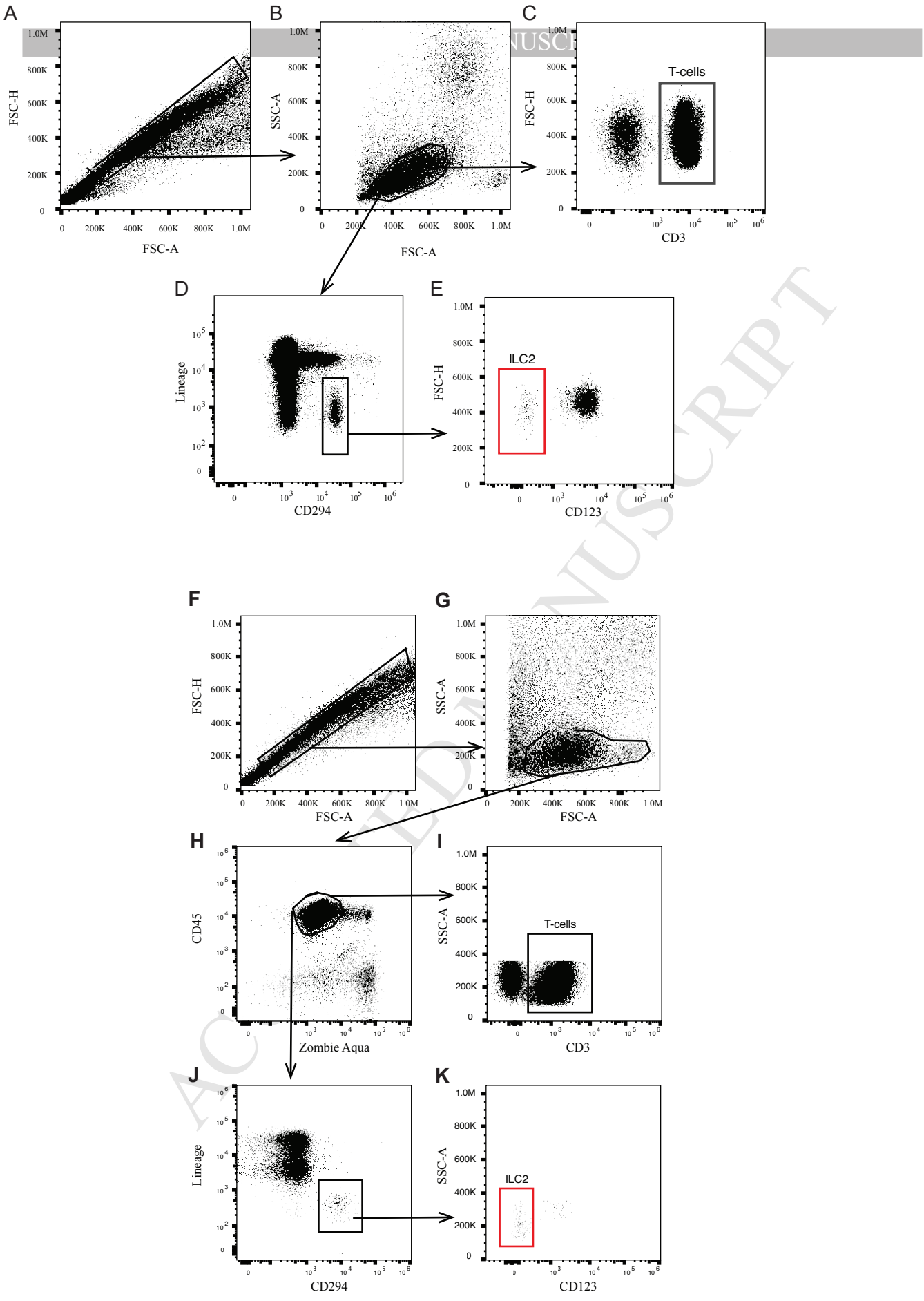
Supplemental Figure 5: Chemokines can be used to stimulate human blood cells in a time dependent manner. A) $CD3^+$ T-cells and **B**) $Lineage^-$, $CD123^-$, $CD294^+$ ILC2s were identified from within a single cell, lymphocyte gate. **C**) CCR4 expression on T-cells (Grey) and ILC2s (Red) with gates used to select positive and negative populations overlaid. Phalloidin staining was analysed for **D**) $CCR4^-$ T-cells, **E**) $CCR4^+$ T-cells and **F**) $CCR4^+$ ILC2s at the indicated time points. Receptor response was determined for **G**) chemokine receptor positive T-cells and **H**) chemokine receptor positive ILC2s at each time point by normalising the phalloidin mean fluorescence intensity to that observed on $CCR4^-$ cells. (Data shown are mean ± SEM of 5 independent donors).

Supplemental table 1: Taqman probes used in this study.

Gene name	**Probe Id**	**Gene name**	**Probe Id**
CCR2	Hs00356601_m1	CCR7	Hs01013469_m1
CCR3	Hs00266213_s1	CCR10	Hs00706455_s1
CCR4	Hs00356611_s1	CXCR4	Hs00237052_m1
CCR5	Hs00152917_m1	CXCR5	Hs00540548_s1
CCR6	Hs01890706_s1	CXCR6	Hs01890898_s1

Supplemental Table 2: Potency (pEC50) and maximal (Emax) responses observed in ILC2s and T-cells following 10s stimulation with the indicated receptor: ligand combinations. Data are mean ± SEM of experiments from at least 5 independent donors.

	CCR2:CCL2		**CCR4:CCL17**		**CCR5:CCL3L1**		**CCR6:CCL20**	
	ILC2	T-cell	ILC2	T-cell	ILC2	T-cell	ILC2	T-cell
pEC50	8.4 ±0.2	8.0 ±0.2	9.6 ±0.2	9.6 ±0.2	8.1 ±0.4	7.7 ±0.2	8.6 ±0.3*	7.5 ±0.1
Emax	1.3 ±0.03	1.2 ±0.02	1.5 ±0.04***	1.2 ±0.02	1.2 ±0.06**	1.4 ±0.04	1.4 ±0.04**	1.6 ±0.05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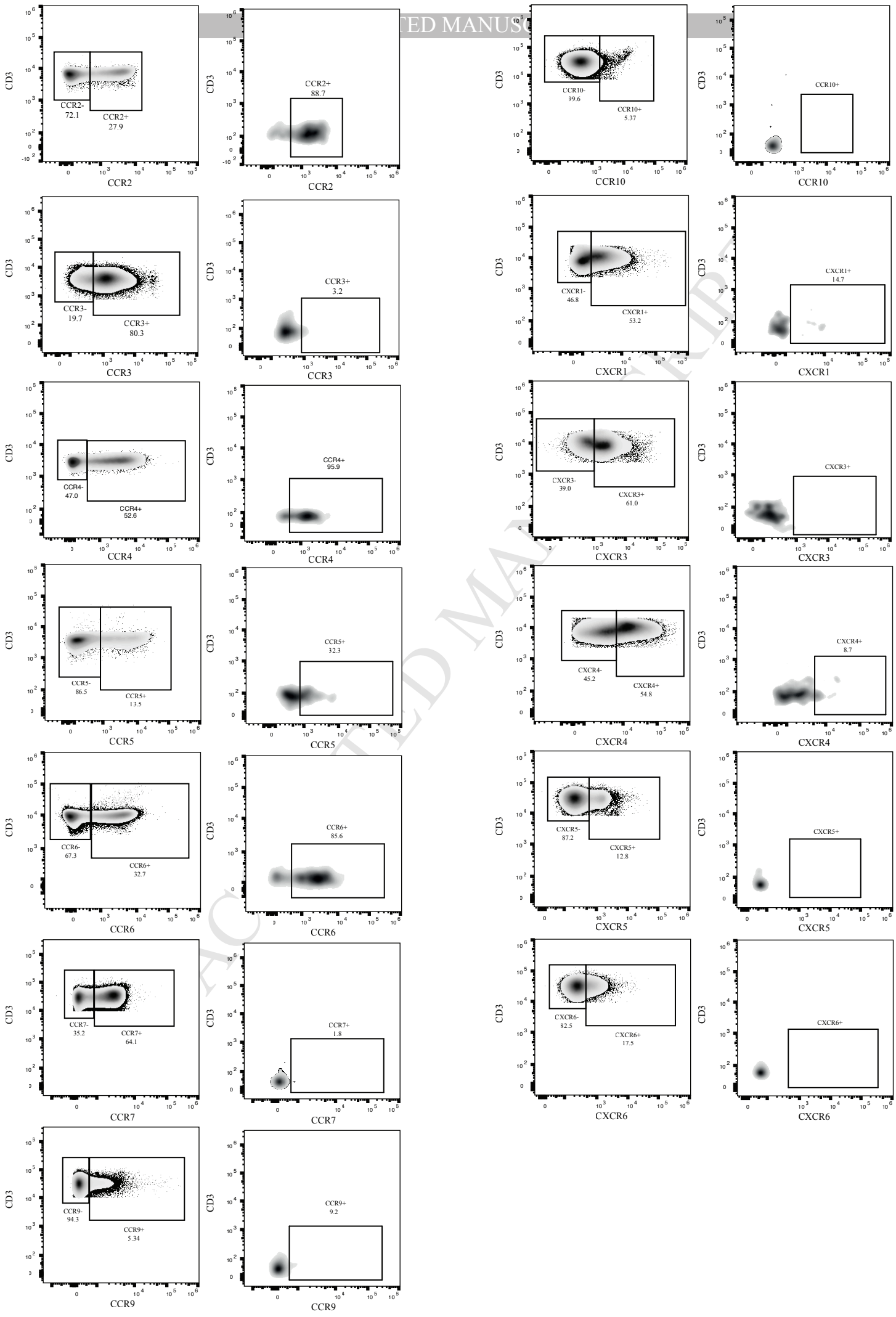

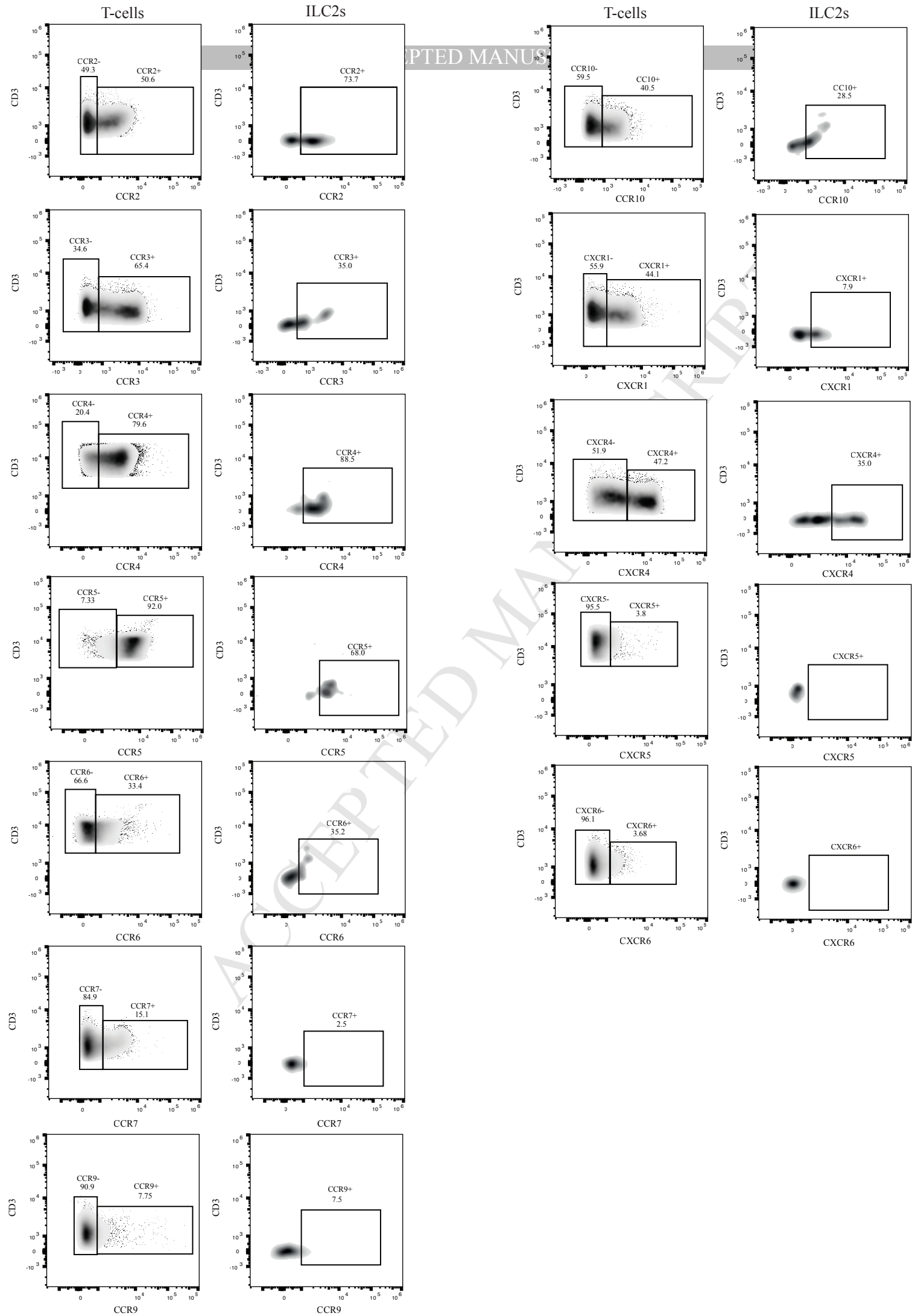

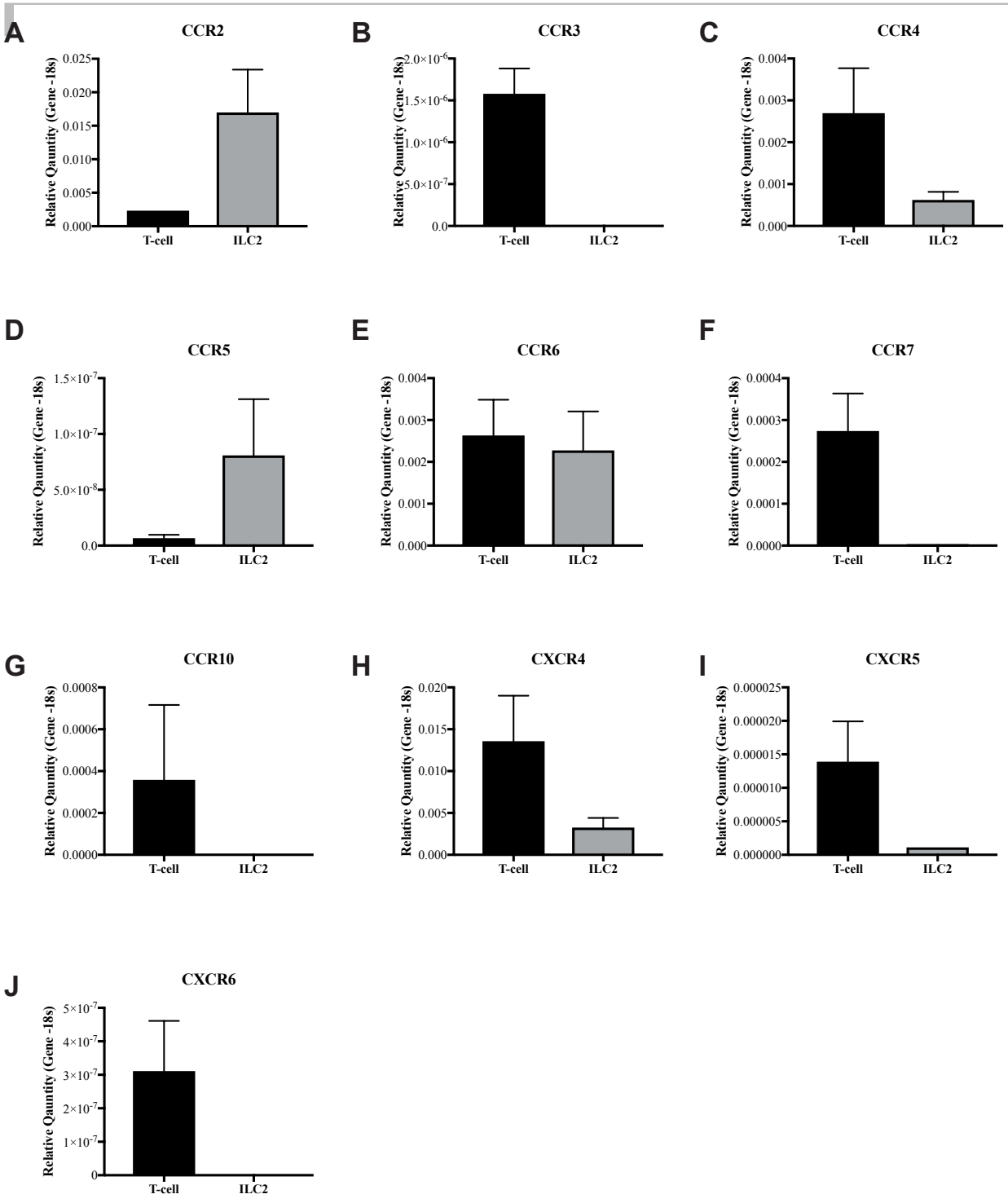
A
CCR2
Relative Qauntity (Gene -18s)
0.025
0.020
0.015
0.010
0.005
0.000
T-cell
ILC2
B
CCR3
Relative Qauntity (Gene -18s)
2.0×10-6
1.5×10-6
1.0×10-6
5.0×10-7
0.0
T-cell
ILC2
C
CCR4
Relative Qauntity (Gene -18s)
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0.003
0.002
0.001
0.000
T-cell
ILC2
D
CCR5
Relative Qauntity (Gene -18s)
1.5×10-7
1.0×10-7
5.0×10-8
0.0
T-cell
ILC2
E
CCR6
Relative Qauntity (Gene -18s)
0.004
0.003
0.002
0.001
0.000
T-cell
ILC2
F
CCR7
Relative Qauntity (Gene -18s)
0.0004
0.0003
0.0002
0.0001
0.0000
T-cell
ILC2
G
CCR10
Relative Qauntity (Gene -18s)
0.0008
0.0006
0.0004
0.0002
0.0000
T-cell
ILC2
H
CXCR4
Relative Qauntity (Gene -18s)
0.020
0.015
0.010
0.005
0.000
T-cell
ILC2
I
CXCR5
Relative Qauntity (Gene -18s)
0.000025
0.000020
0.000015
0.000010
0.000005
0.000000
T-cell
ILC2
J
CXCR6
Relative Qauntity (Gene -18s)
5×10-7
4×10-7
3×10-7
2×10-7
1×10-7
0
T-cell
ILC2